

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number
WO 03/057723 A2

- (51) International Patent Classification⁷: **C07K 7/00**
- (21) International Application Number: **PCT/GB03/00045**
- (22) International Filing Date: **9 January 2003 (09.01.2003)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
0200479.4 9 January 2002 (09.01.2002) **GB**
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- (81) Designated States (*national*): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.**
- (84) Designated States (*regional*): **ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).**
- Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv)) for US only*
- Published:**
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **FLUOROGENIC PROTEASE SUBSTRATES**

(57) **Abstract:** Fluorogenic protease substrates are provided that comprise a peptide doubly labelled via thiol groups of the peptide with an alkyleneamidotetramethylrhodamine (alkyleneamido-TMR) group. Preferred substrates are doubly labelled with substantially pure 5-methyleneamido-TMR or 6-methyleneamido-TMR. Methods of preparing the substrates are provided, which comprise reacting the unlabelled peptide with haloalkylamido-TMR (preferably iodoacetamido-TMR). More generally, fluorogenic protease substrates are also provided which comprise a peptide doubly labelled with the same rhodamine derivative, where the two labels, and their linkages to the peptide, are substantially isomerically identical. Also provided are related methods for assaying protease activity in a sample, kits for use in such methods, and solid supports bearing the substrates of the invention.